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A Non-Radioactive PCR Enzyme-Immunoassay Enables a Rapid Identification of HPV 16 and 18 in Cervical Scrapes After GP5+/6+ PCR

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In previous studies, general primer mediated PCR (GP5+/6+ PCR) was applied successfully to detect a broad spectrum of human papillomaviruses (HPV) in cervical scrapes. In order to facilitate PCR based HPV detection and typing, a colourimetric microtitre plate based hybridisation assay was developed. The method utilised one biotinylated primer (bio-GP6+) in the GP-PCR. Biotinylated PCR products were captured on streptavidin coated microtitre plates, denaturated and hybridised to digoxigenin (DIG) labelled HPV specific internal oligo probes. The DIG labelled hybrids were detected using an enzyme immunoassay (EIA). Since HPV 16 and 18 are the most common HPV types found in cervical carcinomas, this approach was initiated for these two types. Cross-hybridisation reactions were not detected when the specificity of this PCR-EIA for HPV 16 and 18 was tested on a panel of 20 different HPV genotypes. The sensitivity of the assay was found to be between 10 and 100 HPV 16 and 18 viral genomes in a background of 100 ng cellular DNA. This was similar to the detection limit of Southern blot analysis of PCR products with radioactively labelled oligonucleotides. A group of cytomorphologically normal (n = 89) and abnormal (n = 96) cervical scrapes were composed of HPV 16 and HPV 18 positive and HPV negative scrapes. All HPV 16 and 18 positive smears were detected by PCR-EIA. These results indicate that PCR-EIA has the potential for a rapid and sensitive HPV DNA test for day-to-day routine examination of cervical scrapes.

KEY WORDS: HPV 16/18, PCR-EIA, cervical scrapes

INTRODUCTION

Several polymerase chain reaction (PCR) assays have been developed for sensitive and rapid detection of HPV DNA in cervical scrapes [for review see Walboomers et al., 1994]. In the past, consensus or general primer mediated PCRs (GP-PCR) were applied for detection of a broad spectrum of genital HPVs by a single PCR assay [Manos et al., 1989; Gregoire et al., 1989; Snijders et al., 1990; De Roda Husman et al., 1995]. Subsequent typing into individual HPVs could then be carried out in several ways. First, by application of additional HPV type specific PCR (TS-PCR) with subsequent oligo probe hybridisation [Van den Brule et al., 1990], or hybridisation of the GP-PCR products themselves with internal oligo probes [Manos et al., 1989; Jacobs et al., 1995]. Alternatively, nucleotide sequencing [Smits et al., 1992] or restriction fragment length polymorphism analysis of PCR products [Lungu et al., 1992] could be undertaken.

Much effort has been devoted to improve PCR based HPV detection for routine examination of large numbers of cervical scrapes. The easy application of GP-PCR directly to crude cervical cell suspensions [Van den Brule et al., 1990], omitting the laborious DNA purification steps, has already been shown to make PCR more suitable for large HPV DNA screening programmes [Eluf-Neto et al., 1994; De Roda Husman et al., 1994]. However, the practical application of daily HPV DNA testing of large numbers of cervical scrapes requires detection methods that are not only specific and sensitive but also simple and rapid.

The most promising alternative to simplify HPV DNA detection by PCR is to analyse PCR generated products with an enzyme immunoassay (EIA). Several EIA ap-

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proaches using hybridisation with oligo probes have been described to detect amplified DNA from microorganisms in microtitre plates [Tada et al., 1992; Conway et al., 1992; King and Ball, 1993; Ossewaarde et al., 1994; Lungu et al., 1995]. Most of these procedures are based on the use of a biotinylated primer to bind amplified DNA to streptavidin coated microtitre wells. Using this capturing strategy we developed a PCR-EIA which allows a rapid identification of HPV DNA in cervical scrapes after HPV general primer GP5+/6+ mediated PCR [De Roda Husman et al., 1995]. Since HPV 16 and HPV 18 are the most commonly found HPV types in cervical carcinomas, this study has focused on these two HPVs. For evaluation, Southern blot analysis of GP5+/bio-GP6+ PCR products using radioactive oligo probes was compared to PCR-EIA. Therefore, both methods were applied to a group of cytomorphologically normal (n = 89) and abnormal (n = 96) cervical scrapes, previously shown to be HPV negative or to contain either HPV 16 or HPV 18.

MATERIALS AND METHODS

Viral DNA Clones

HPV clones were used to compose an HPV test panel containing the following HPV types: 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 54, 56, and 58, 59, 66, 68. HPV clones of types 6b, 11, 16 and 18 were kindly provided by Drs. H. zur Hausen and L. Gissmann (Heidelberg, Germany), HPV 40 and 68 (ME180) by Dr. E.-M. de Villiers (Heidelberg, Germany), HPV 31 by Dr. A. Lorincz (Gaithersburg, MD), HPV 33, 39, 42, 54 and 66 by Dr. G. Orth (Paris, France), HPV 45 by Dr. K. V. Shah (Baltimore, MD), HPV 51 by Dr. G. Nuovo (New York, NY) and HPV 58 and 59 by Dr. T. Matsukura (Tokyo, Japan). Cloned HPV types 35, 43 and 56 were obtained from the ATTCC (Rockville, MD). The GP-PCR product of HPV type 52 was cloned in our laboratory from a cervical scrape and was verified by sequence comparison [De Roda Husman et al., 1994]. DNA was extracted for PCR using a Qiagen-Pack 500 kit (Qiagen, Germany) according to the manufacturer's protocol.

Clinical Specimens

Cervical scrapes were derived in 1993 from women attending the Department of Gynaecology, Free University Hospital, Amsterdam, The Netherlands. A group of cytomorphologically normal (n = 89) and abnormal (n = 96) cervical scrapes were selected, shown previously to be HPV negative or to be HPV positive after GP5+/6+ PCR. The HPV positive scrapes contained either HPV 16 or HPV 18 as determined by HPV type specific PCR. The cytomorphological classification of the cervical scrapes were carried out according to a modified KOPAC classification used in The Netherlands [Vooijs, 1987].

For cytological analysis and HPV detection, two cervical scrapes were taken in each case. The first brush was used for routine cytomorphological classification. For HPV detection, the remaining material of the first brush and material from the second scrape were placed into 5 ml PBS containing 0.05% Merthiolate. After vortexing vigorously, the suspensions were centrifuged for 10 min at 3,000*g*. The cell pellets were resuspended in 1 ml 0.01 M Tris (pH 7.5) and frozen at −20°C. For PCR, 10 μl aliquots were boiled for 10 min at 100°C, cooled on ice and centrifuged for 1 min at 3,000*g* before addition of the PCR mixture.

DNA Polymerase Chain Reaction

Either isolated DNA from cloned HPVs or 10 μl of pretreated crude cell suspensions of cervical scrapes were subjected to standard GP5+/GP6+ PCR as described [De Roda Husman et al., 1995] except that the GP6+ primer was biotinylated at the 5′ end during oligonucleotide synthesis (bio-GP6+). Briefly, the GP-PCR was carried out in 50 μl of PCR solution containing 50 mM KCl, 10 mM Tris HCl pH 8.3, 200 μM of each dNTP, 3.5 mM $MgCl_2$, 1 unit of thermostable DNA polymerase (AmpliTaq; Cetus Corporation, Emeryville, CA, USA), and 25 pmol of each of the GP5+/bio-GP6+ primers. The mixture was overlaid with several drops of paraffin oil to prevent evaporation and incubated for 5 min at 96°C for DNA denaturation. Forty cycles of amplification were carried out using a PCR processor (Biomed, Krefeld, FRG). Each cycle included a denaturation step at 96°C for 1 min, followed by a primer annealing step at 40°C for 2 min and a chain elongation step at 72°C for 1.5 min. To ensure a complete extension of the amplified DNA the final elongation step was prolonged by 4 min. Five microliters and 10 μl of the biotinylated GP-PCR products were used for EIA and Southern blotting, respectively.

Labelling of Oligonucleotides

Type specific oligonucleotides [Jacobs et al., 1995] for HPV 16 (5′-GTCATTATGTGCTGCCATATCTACTTCAGA-3′) and HPV 18 (5′-TGCTTCTACACAGTCTCCTGTACCTGGGCA-3′) were synthesized by the methoxy-phosphoramidite method (Pharmacia, Uppsala, Sweden). The oligonucleotides were 5′[γ-^{32}P]dATP end-labelled by means of T4 polynucleotide kinase (Promega, USA). The oligonucleotides were also end-labelled with digoxigenin-11-ddUTP (DIG) using terminal transferase according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Removal of free digoxigenin molecules was carried out by precipitation with 4 M $LiCl_2$, after which pellets were dried and dissolved in 20 μl sterile H_2O. One microliter of serial tenfold dilutions of the oligo probe and the DIG labelled control oligonucleotide (Boehringer-Mannheim) were spot blotted onto a nylon membrane to estimate the labelling efficiency. The oligo probes were visualised using the chemiluminescent substrate disodium-tricyclo phenyl phosphate (CSPD) according to the manufacturer's protocol (Boehringer-Mannheim). Briefly, the spot blots were rinsed in 0.1 M Maleic acid/0.15 M NaCl (pH 7.5) for 1 min at ±20°C (room temperature; RT). Subsequently, the spot blots were placed in a 1% blocking buffer (Boehringer-Mannheim) for 30 min at RT. Alkaline phosphatase conjugated anti-DIG antibodies was added in a final concentration of 75 mU/ml and incubated for 30 min at RT. The spot blots were washed

three times for 15 min with 0.1 M Maleic acid/0.15 M NaCl/0.3% Tween 20 and equilibrated afterwards in chemiluminescence buffer (50 mM $MgCl_2$/0.1 M Tris-HCl/0.1 M NaCl, pH 9.5) for 5 min at RT. Thereafter, disodium-tricyclo phenyl phosphate (CSPD) was added in a final concentration of 250 μM. Finally, after 5 min incubation at RT, the spot blots were sealed in plastic bags and chemiluminescent detection was carried out for 5 to 15 min with Kodak Royal X-omat film and intensifying screens. The final concentration of the DIG labelled oligo probes was estimated by comparing the hybridisation signal of the spots to the hybridisation signals of the spots from the control DIG labelled oligonucleotide.

Enzyme Immunoassay

Enzyme immunoassays were carried out in streptavidin coated microtitre plates (Labsystems, Helsinki, Finland). Washings were performed at RT. The EIA procedure used biotinylated PCR products that had been captured on streptavidin coated microtitre wells. Subsequently, the wells were washed and the captured DNA was denatured by alkaline treatment. Wells were washed again followed by hybridisation with DIG labelled oligo probes. Unbound probe was removed by washings. The ultimate hybrids were then detected using anti-DIG antibodies conjugated with alkaline phosphatase, and after removing the unbound polyclonal antibodies, p-nitrophenyl phosphate (pNPP) was added as substrate. After incubation at 37°C, the optical density (OD) was measured at 405 nm against 620 nm with an Anthos htII reader (Labtec, Salzburg, Austria). To optimize this EIA procedure for the detection of HPV 16 and HPV 18 after GP-PCR, several variables as described in the literature were tested in comparative reconstruction experiments on cloned HPV 16 DNA diluted in human placental DNA. Briefly, different amounts of biotinylated PCR products were tested for adequate capture. Also several hybridisation- and wash buffers [Suzuki et al., 1993; King and Ball, 1993; Ossewaarde et al., 1994] were compared as well as different hybridisation temperatures [Tada et al., 1992; Van der Vliet et al., 1993; Ossewaarde et al., 1994]. In addition, several probe concentrations [Suzuki et al., 1993; Ossewaarde et al., 1994] were tested and, finally, different substrate incubation times ranging from 1–4 hr to overnight were evaluated.

The specificity of the PCR-EIA using oligo probes for HPV 16 or 18 was determined on a panel of GP-PCR products derived from 1 ng pHPV input DNA of the following HPV types 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 54, 56, 58, 59, 66, 68 and 10 pg of the GP-PCR product of HPV type 52. The sensitivity of the assay was determined by GP5+/bio-GP6+ PCR using tenfold dilutions of HPV 16 and 18 plasmid input DNA ranging from 10 pg down to 10 ag. In each reaction 100 ng human placental DNA was present as background. As PCR negative controls (PNC) PCR mixture containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 3.5 mM $MgCl_2$, 200 μM of each dNTP, 25 pmol of the GP5+ and bioGP6+ primers, and 1 U of thermostable DNA polymerase) with 100 ng human placental DNA were used.

Southern Blot Analysis of GP-PCR Products

Ten microliters of the biotinylated PCR products was layered on 1.5% agarose gels and transferred onto positively charged nylon membranes (Qiabrane, Qiagen, Hildeu) by capillary blotting in 0.5 N NaOH, 0.6 M NaCl, respectively.

Membranes were prehybridised for 2 hr at 55°C in hybridisation solution (0.5 M sodium phosphate [pH 7.4], 7% sodium dodecyl sulphate [SDS], 1 mM EDTA). Afterwards [γ-^{32}P]dATP labelled HPV 16 and 18 specific oligonucleotides were added and the hybridisations were carried out at the same temperature overnight. Subsequent washings were carried out three times at 55°C for 30 min using 3 × SSC/0.5% SDS. Autoradiography was carried out overnight at −70°C with Kodak Royal X-omat film and intensifying screens [Van den Brule et al., 1990].

RESULTS

Optimization of EIA Detection of GP5+/bio-GP6+ PCR Products

As outlined in Materials and Methods, several variables such as the amounts of PCR products for capture, probe concentration, and composition of hybridisation buffer were first tested in comparative experiments on cloned HPV 16 DNA diluted in human placental DNA to determine the optimal test conditions. The final protocol was obtained as follows. Five microliter aliquots of PCR products per well could be used after which 50 μl 1 × SSC/0.5% Tween 20 (hybridisation buffer) was added. The plates were incubated at 37°C for 1 hr. After four washes with hybridisation buffer, 100 μl 0.2 M NaOH was added for a 15 min denaturation step at RT. After two washings with hybridisation buffer, 50 μl hybridisation buffer containing 10 picomoles/ml DIG labelled HPV specific oligo probe was added. After 1 hr of hybridisation at 37°C the wells were washed twice and 50 μl alkaline phosphatase conjugated anti-DIG antibodies (75 mU/ml hybridisation buffer) was used for incubation at 37°C for 1 hr. The plates were then washed four times with hybridisation buffer and 100 μl pNPP (1 mg/ml 0.2 M Tris) was added. The plates were incubated at 37°C for 1 hr before the ODs were measured. Using a cut-off value of 3 × the OD of the PCR negative controls (PNC) the EIA showed a sensitivity of 1 fg of HPV 16 DNA already after 1 hr of incubation (Table I). However, the most optimal signal-to-noise ratio was obtained after overnight incubation. As shown in Table I, the signal-to-noise ratio for the 1 fg level increased about twofold from 4.2 (OD_{1fg}/OD_{PNC} = 0.265/0.062) at 1 hr pNPP incubation to 8.8 (OD_{1fg}/OD_{PNC} = 1.669/0.189) after O/N incubation.

Specificity and Sensitivity of the Enzyme Immunoassay

After establishing the optimal conditions the specificity of the PCR-EIA was determined on a panel of GP-PCR products derived from HPV genotypes HPV 6, 11,

TABLE I. Optical Density in Relation to Substrate Incubation Time*

Amount of pHPV 16 prior to amplification	Substrate incubation time				
	1 hr	2 hr	3 hr	4 hr	O/N[a]
10 pg	1.647	2.665	2.911	9.999	9.999
1 pg	1.308	2.165	2.527	2.797	9.999
100 fg	1.108	1.851	2.261	2.549	9.999
10 fg	0.783	1.346	1.717	1.941	9.999
1 fg	0.265	0.458	0.577	0.661	1.669
100 ag	0.103	0.168	0.232	0.359	0.496
10 ag	0.065	0.082	0.093	0.162	0.207
PNC[b]	0.062	0.076	0.086	0.120	0.189

*Optical densities were measured after incubations in 1 × SSC/0.5% Tween 20 at 37°C.
[a]Overnight substrate incubation.
[b]PCR negative control.

16, 18, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 54, 56, 58, 59, 66, and 68. No cross-hybridisation of the HPV 16 and 18 oligo probes with 20 of the remaining types could be demonstrated, which is in agreement with earlier findings using a radioactive filter hybridisation assay [Jacobs et al., 1995].

The results of the EIA for both HPV 16 and 18 were compared to those obtained on the same PCR products after standard agarose gel electrophoresis and additional Southern blot hybridisation with radioactive oligo probes. After gel electrophoresis and ethidium bromide staining, between 100 fg and 10 fg was detected (Fig. 1A), whereas after additional Southern blot hybridisation the detection rate increased to 1 fg of both HPV 16 and HPV 18 (Fig. 1B). Using a cut-off value of 3 × the OD of the PCR negative control the EIA showed also a sensitivity of 1 fg for both HPV 16 and HPV 18 (Fig. 1C). In addition, OD values after 1 hr substrate incubation by EIA (Table I) were plotted against HPV copy numbers in a graphical manner (Fig. 2). A linear-logarithm relationship in the range from 10 to 10^6 genome equivalents of HPV 16 and 18 (input DNA) and the ODs were observed, after 1 hr following substrate addition.

Comparison of EIA With Southern Blot Analysis of GP-PCR Products Using Radioactive Oligo Probes on Cervical Scrapes

To assess the value of the PCR-EIA for the identification of HPV 16 and HPV 18 DNA, the method was applied to 185 selected cervical scrapes of different PAP classes and compared with the results obtained using Southern blot analysis of GP5+ /bio-GP6+ PCR products after hybridisation with HPV 16 or HPV 18 specific oligo probes. The results are summarised in Table II. Independent of cytological classification, all HPV negative scrapes (n = 39) show to be negative by the EIA. For both HPV 16 and HPV 18 detection, all Southern blot HPV positive scrapes (n = 147) were also positive by EIA after overnight reading. Cervical scrapes that were scored already positive after gel electrophoresis and ethidium bromide staining of the GP-PCR products gave after overnight substrate incubation by the EIA ODs ranging from 1.698 to 9.999 (Table II; part enzyme immunoassay; columns 1 and 3: gel [OD]). The ODs from the scrapes negative at the gel level but only HPV positive after Southern blot hybridisation varied from 0.402 to 0.786 after overnight incubation (Table II; part enzyme immunoassay; columns 2 and 4: blot [OD]). Using clinical specimens, cross-hybridisations between HPV 16 and 18 were not found using EIA, thereby confirming its specificity.

DISCUSSION

The evidence linking genital tract human papillomavirus infections with cervical cancer has been derived from epidemiological [Munoz et al., 1992; Schiffmann et al., 1993; Eluf-Neto et al., 1994] and molecular biological studies [Zur Hausen, 1994]. Currently, more than 70 HPV genotypes have been molecularly cloned [De Villiers, 1994] and at least 30 HPV types have been isolated from the genital tract. A subset of 15 different genital HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66 and 68) are associated with cervical carcinomas and carcinomas in situ [Lorincz et al., 1992; De Roda Husman et al., 1994; Bosch et al., 1995]. Therefore, these high risk types may be relevant for incorporation in HPV DNA detection tests for routine cervical cancer screening and management of patients with cervical lesions [Meijer et al., 1992; Walboomers et al., 1994; Cuzick et al., 1995]. The application of a single general primer mediated HPV PCR followed by typing using cocktails of high risk HPV type specific oligo probes [Jacobs et al., 1995] in a colourimetric microtitre plate hybridisation assay would therefore be most appropriate. In an effort to develop such an HPV group specific PCR-EIA system we initially focused on the identification of HPV 16 and 18 since these two viruses are found most commonly in cervical carcinomas.

For a suitable EIA method, the capture conditions for the biotinylated PCR products and the hybridisation- and staining conditions should be optimised. In this context, it appeared that in our hands home-made streptavidin coated microtitre plates yielded high levels of background. Commercially available plates showed much more specific and reproducible results, probably due to standardised coating and blocking procedures. For this reason, commercial microtitre plates were used throughout the study. In addition, it appeared that the

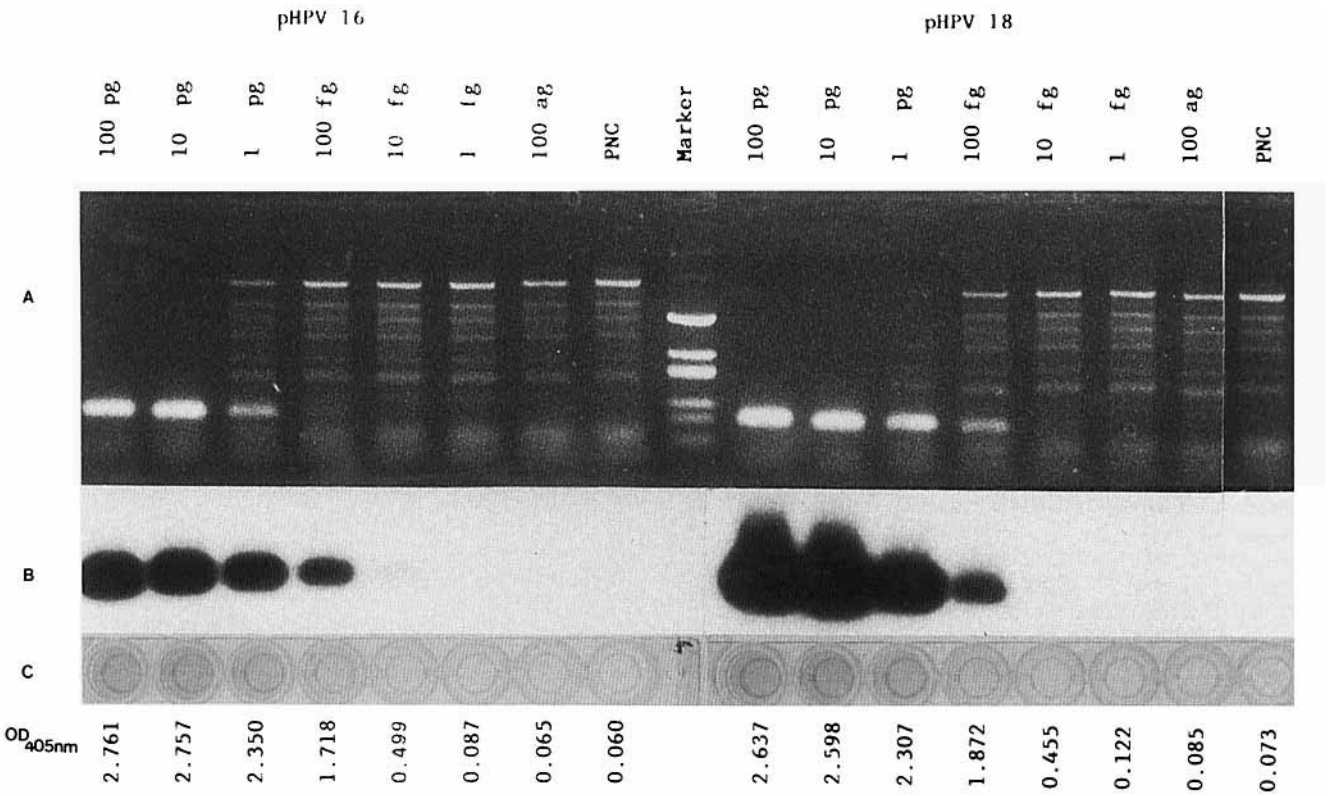

Fig. 1. Comparison of sensitivity of gel electrophoresis **(A)**, oligo filter hybridisation **(B)** and EIA with optical densities read after 3 hr substrate incubation **(C)** to detect HPV 16 and 18 specific GP5+/bio-GP6+ PCR products. Serial tenfold dilutions of pHPV 16 and pHPV 18 target DNA are indicated above the lanes. Due to photographic reduction the 1 fg signal is poorly visible. For photographic reasons, the EIA results of 3 hr substrate incubation are shown.

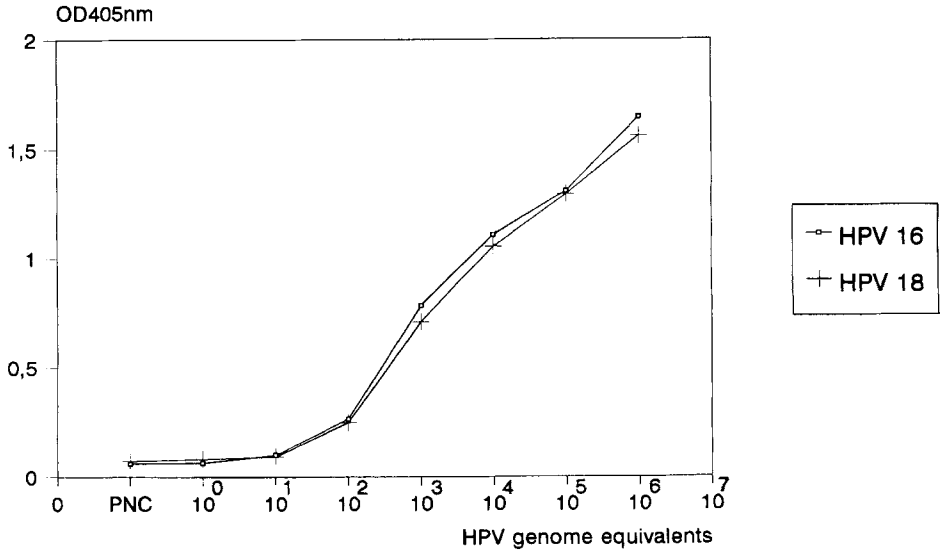

Fig. 2. Relationship between HPV genome equivalents and optical densities obtained after 3 hr of pNPP substrate incubation.

TABLE II. Comparison of Southern Blot Hybridization (SB) and PCR-EIA of GP PCR Products for the Detection of HPV 16 and HPV 18 in Cervical Smears

				HPV positive smears							
				SB analysis of PCR prod.				Enzyme-immunoassay			
		HPV neg	HPV pos	HPV 16		HPV 18		HPV 16		HPV 18	
Cytology	n	n	n	gel	blot	gel	blot	gel (OD)	blot (OD)	gel (OD)	blot (OD)
Normal (Pap I/II)	89	30	59	41	1	15	2	41 (1.708/9.999)	1 (0.730)	15 (1.698/9.999)	2 (0.730/0.775)
Mild dysplasia (Pap IIIa)	47	7	40	30	3	6	1	30 (2.262/9.999)	3 (0.402/0.531)	6 (1.835/9.999)	1 (0.786)
Severe dysplasia Pap (IIIb)	31	2	29	26		3		26 (1.786/9.999)		3 (2.650/9.999)	
Carcinoma in situ (Pap IV)	18		18	14	1	3		14 (1.747/9.999)	1 (0.747)	3 (2.431/9.999)	

Gel: smears already positive after gel electrophoresis and ethidium bromide staining. Blot: smears positive only after additional Southern blot hybridization of the PCR products. Optical densities were obtained after overnight substrate incubation. The lowest and highest measured ODs are indicated between brackets, respectively.

use of 5–10 μl GP-PCR product gave already optimal EIA results. Furthermore, it became clear that one buffer (1 × SSC/0.5% Tween 20) could be used throughout the whole EIA procedure, which simplifies the method and facilitates future automation. In addition, it is stressed that freshly made washing buffer is essential for maintaining low background signals. Finally, an optimised protocol could be obtained as described. This study showed that the optimised EIA method was highly specific, since HPV 16 and 18 specific oligo probes did not cross-hybridize with PCR products generated from 20 other HPV genotypes. Furthermore, the sensitivity of the optimised PCR-EIA was between 10 and 100 copies of the viral genome and was comparable with data obtained by other investigators using either a radioactive hybridisation procedure of PCR products (see Fig. 1) [De Roda Husman et al., 1995] or EIA based detection methods for other micro-organisms [Allen et al., 1995; Van der Vliet et al., 1993]. However, the PCR-EIA in this study has the potential of greater sensitivity, as shown by the relative high ODs at the 1 fg level of HPV 16 and 18 DNA in comparison to Southern blot hybridisation of the GP-PCR products. This could be due to fewer manipulation steps involved, particularly the blotting procedure. In addition, improvements may be obtained using beads coated with streptavidin [Suzuki et al., 1993]. Moreover, the coating of beads with streptavidin may be easier to standardise and a higher density of streptavidin can be obtained due to a larger surface. The use of beads for HPV detection is presently under investigation.

Since it seems that virus load is informative for the underlying lesions in scrapes with mild dysplasia [Cuzick et al., 1995] quantitative aspects are also important. The PCR-EIA can be used potentially semi-quantitatively to assess the relative amount of HPV DNA in cervical scrapes, since a linearity between the amount of input DNA and the ODs was demonstrated in the range of 10 to 10^6 genome equivalents after 1 hr pNPP incubation (Fig. 2). These 1 hr readings are preferred in practice for semi-quantification of the PCR products since longer substrate incubation times resulted in a smaller linear range (see also Table I).

We demonstrated the value of the optimised PCR-EIA for the detection of HPV 16 and HPV 18 DNA using a selected group of cervical scrapes. All HPV 16 and 18 positive samples were positive by the PCR-EIA, independent of their cytological classification. Although after 1 hr substrate incubation time, weakly HPV positive scrapes could be detected, overnight incubation resulted in more evident results (see also Table I; 1 fg HPV 16 DNA). This is also substantiated by the observation that specific PCR signals at the gel level show higher ODs compared to the OD values of PCR signals seen only after additional Southern blot hybridisation of GP-PCR products. Based on all the results, it is advisable to read the OD value at two time points in practice either for quantitative or qualitative analysis of PCR products.

Although a 100% agreement was observed between EIA and Southern blot analysis it is worth mentioning that in manually performed enzyme immunoassays the results may be influenced by the many manipulating steps involved (e.g. pipetting and washing steps) which can lead to errors. Therefore, automation of the EIA steps is recommended for routine practice. In addition, the optimised EIA based detection method has the advantage of producing numerical data and is not dependent on subjective interpretation.

Finally, HPV detection in cervical scrapes in combination with cytology for improving cervical cancer screening and treatment of patients with cervical intraepithelial neoplastic (CIN) disease has been proposed recently [Meijer et al., 1992; Walboomers et al., 1994]. This is based on the relative low HPV prevalence found in normal cytology in women aged 35–55 years (cervical cancer screening population) in The Netherlands [Melkert et al., 1993] and the recent demonstration that persistence of oncogenic HPVs is a marker for progression of CIN disease [Remmink et al., 1995]. Since in the last study progression was not only associated with HPV 16 and HPV 18, but also other high risk HPVs, a proper PCR-EIA should include all known high risk HPV types. A

step in that direction has recently been made by Lungu et al. [1995], who included nine high risk HPV types in the PCR-EIA. We are currently extending our PCR-EIA to 15 high risk HPV types.

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REFERENCES

Allen RD, Pellett PhE, Stewart JA, Koopmans M (1995): Nonradioactive PCR-enzyme linked immunosorbent assay method for detection of human cytomegalovirus DNA. Journal of Clinical Microbiology 33:725–728.

Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, Shah KV, Alihonou E, Bayo S, Mockhtar HC, Chicareon S, Daudt A, Delosrios E, Ghadirian P, Kitinya JN, Koulibaly M, Ngelangel C, Tintore LMP, Riosdalenz JL, Sarjadi A, Schneider A, Tafur L, Teyssie AR, Rolon PA, Torroella M, Tapia AV, Wabinga HR, Zatonski W, Sylla B, Vizcaino P, Magnin D, Kaldor J, Greer C, Wheeler C (1995): Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. Journal of the National Cancer Institute 87:796–802.

Conway B, Bechel LJ, Adler KA, D'Aquila RT, Kaplan JC, Hirsch MS (1992): Comparison of spot-blot and microtitre plate methods for the detection of HIV-1 PCR products. Molecular and Cellular Probes 6:245–249.

Cuzick J, Szarewski A, Terry G, Ho L, Hanby A, Maddox P, Anderson M, Kocjan G, Steele ST, Guillebaud J (1995): Human papillomavirus testing in primary cervical screening. The Lancet 345:1533–1536.

De Roda Husman AM, Walboomers JMM, Meijer CJLM, Risse EKJ, Schipper MEI, Helmerhorst TM, Bleker OP, Delius H, van den Brule AJC, Snijders PJF (1994): Analysis of cytomorphologically abnormal cervical for the presence of 27 mucosotropic human papillomavirus genotypes, using polymerase chain reaction. International Journal of Cancer 56:802–806.

De Roda Husman AM, Walboomers JMM, van den Brule AJC, Meijer CJLM, Snijders PJF (1995): The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly concerved sequences improves human papillomavirus detection by polymerase chain reaction. Journal of General Virology 76:1057–1062.

De Villiers EM (1994): Human pathogenic papillomavirus types: an update. In zur Hausen H (ed.): "Human Pathogenic Papillomaviruses." Current Topics in Microbiology and Immunology 186:1–12.

Eluf-Neto J, Booth M, Munoz N, Bosch FX, Meijer CJLM, Walboomers JMM (1994): Human papillomavirus and invasive cervical cancer in Brazil. British Journal of Cancer 69:114–119.

Gregoire L, Arella M, Campione-Piccardo J, Lancaster WD (1989): Amplification of human papillomavirus DNA sequences by using conserved primers. Journal of Clinical Microbiology 27:2660–2665.

Jacobs MV, de Roda Husman AM, van den Brule AJC, Snijders PJF, Meijer CJLM, Walboomers JMM (1995): Group-specific differentiation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. Journal of Clinical Microbiology 33:901–905.

King JA, Ball K (1993): Detection of HIV-1 by digoxigenin labelled PCR and microtitre plate solution hybridisation assay and prevention of PCR carry-over by uracil-N-glycosylase. Journal of Virological Methods 4:67–76.

Lorincz AT, Reid R, Jenson AB, Greenberg MD, Lancaster W, Kurman RJ (1992): Human papillomavirus infection of the cervix: Relative risk associations of 15 common anogenital types. Obstetrics and Gynecology 79:328–337.

Lungu O, Sun XW, Felix J, Richart RM, Silverstein S, Wright TC (1992): Relationship of human papillomavirus type to grade of cervical intraepithelial neoplasia. Journal of American Medical Association 267:2493–2496.

Lungu OX, Sun XW, Wrigth TC, Ferenczy A, Richard RM, and Silverstein S (1995): A polymerase drain reaction-enzyme-linked immunosorbent assay method for detecting human papilloma virus in cervical carcinomas and high-grade cervical cancer precursors. Obstetrics and Gynecology 85:337–342.

Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinski SM (1989): Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells 7:209–214.

Meijer CJLM, van den Brule AJC, Snijders PFJ, Helmerhorst ThJM, Kenemans P, Walboomers JMM (1992): Detection of human papillomavirus in cervical by the polymerase chain reaction in relation to cytology: Possible implication for cervical cancer screening. In Munoz N, Bosch FX, Shah KV, Maheus A (eds.): "The Epidemiology of Cervical Cancer and Human Papillomavirus." IARC Scientific Publication, pp 271–280.

Melkert PWJ, Hopman E, van den Brule AJC, Risse EKJ, van Diest PJ, Bleker OP, Helmerhorst T, Schipper MEI, Meijer CJLM, Walboomers JMM (1993): Prevalence of HPV in cytomorphologically normal cervical scrapes as determined by the polymerase chain reaction is age-dependent. International Journal of Cancer 53:1-5.

Munoz N, Bosch FX. Sanjose, Tafur L, Izarzugaza I, Gili M, Viladiu P, Navarro C, Martos C, Ascunce N, Gonzaliz LC, Kaldor JM, Guerrero E, Lorincz AT, Santamaria M, Alonso de Ruiz R, Aristizabal N, Shah K (1992): The causal link between human papillomavirus and invasive cervical cancer: A population-based case-control study in Colombia and Spain. International Journal of Cancer 52:743–749.

Ossewaarde JM, Rieffe M, van Doornum GJJ, Henquet CJM, van Loon AM (1994): Detection of amplified Chlamydia trachomatis DNA using a microtitre plate-based enzyme immunoassay. European Journal of Clinical Microbiology and Infectious Disease 13:732–739.

Remmink AJ, Walboomers JMM, Helmerhorst TJM, Voorhorst FJ, Roozendaal L, Risse EKJ, Meijer CJLM, Kenemans P (1995): The presence of persistent high risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: Natural history up to 36 months. International Journal of Cancer 61:306–311.

Schiffmann MH, Bauer HM, Hoover RH, Glass AG, Cadell DM, Rush BB, Scott DR, Sherman ME, Kurman RJ, Wacholder S, Stanton CK, Manos MM (1993): Epidemiological evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasias. Journal of the National Cancer Institute 85:958–964.

Smits HL, Tieben LM, Tjong-A-Hung SP, Jebbink MF, Minnaar RP, Jansen CL, ter Schegget J (1992): Detection and typing of human papillomaviruses in fixed and stained archival cervical scrapes by a consensus polymerase chain reaction and direct sequence analysis allowing the identification of a broad spectrum of human papillomavirus types. Journal of General Virology 73:3263–3268.

Snijders PJF, van den Brule AJC, Schrijnemakers HFJ, Snow G, Meijer CJLM, Walboomers JMM (1990): The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. Journal of General Virology 71:173–181.

Suzuki K, Okamoto N, Kano T (1993): Colorimetric detection for PCR amplified HIV-I DNA using magnetic beads. Journal of Virological Methods 41:341–350.

Tada J, Ohashi T, Nishimura N, Ozaki H, Fukushima S, Takano J, Nishibuchi N, Takeda Y (1992): Non-isotopic microtitre plate-based assay for detecting products of polymerase chain reaction amplification: Application to detection of the tdh of Vibrio parahaemolyticus. Molecular and Cellular Probes 6:489–494.

Van den Brule AJC, Meijer CJLM, Bakels V, Kenemans P, Walboomers JMM (1990): Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type-specific polymerase chain reaction. Journal of Clinical Microbiology 28: 2739–2743.

Van der Vliet GME, Hermans CJ, Klatser PR (1993): Simple colorimetric microtitre plate hybridisation assay for detection of amplified Mycobacterium leprae DNA. Journal of Clinical Microbiology 31:665–670.

Vooijs GP (1987): De advisering bij afwijkende bevindingen van cytologisch onderzoek van de cervix uteri. Nederlands Tijdschrift voor Geneeskunde 131:1662–1663.

Walboomers JMM, de Roda Husman AM, van den Brule AJC, Snijders PJF, Meijer CJLM (1994): Detection of genital human papillomavirus infections: Critical review of methods and prevalence studies in relation to cervical cancer. In Stern PL, Stanley M (eds): "Human Papillomaviruses and Cervical Cancer." New York: Oxford University Press, pp 41–71.

Zur Hausen H (1994): Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. In Zur Hausen H (ed.): "Human Pathogenic Papillomaviruses." Heidelberg, New York: Springer Verlag, pp 133–156.